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Combined Use of a Water-Insoluble Chemical Delivery System and a Metabolic Activation System in Whole Embryo Culture

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16. ABSTRACT

An integrated water insoluble chemical delivery/metabolic activation/rat embryo culture system is described. In initial studies corn oil was used as the solvent and diallate as the substrate. Increasing concentrations of diallate dissolved in corn oil caused embryonic growth retardation in vitro. The presence of a metabolic activity system did not potentiate the effects of diallate in corn oil.

The rat hepatic microsomal monooxygenase system was enzymatically active even in the presence of a high concentration of corn oil in the rat serum incubation media. Thus, the failure of the metabolic activation system to potentiate the toxicity diallate in corn oil is not due to an enzymatic deficiency of the hepatic metabolic activation system.

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COMBINED USE OF A WATER-INSOLUBLE CHEMICAL DELIVERY SYSTEM AND A METABOLIC ACTIVATION SYSTEM IN WHOLE EMBRYO CULTURE

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An integrated water-insoluble chemical delivery/metabolic activation/rat embryo culture system is described. Increasing concentrations of diallate dissolved in corn oil caused dose-related embryonic growth retardation in vitro. The presence of a metabolic activating system did not potentiate the effects of diallate in corn oil. The rat hepatic microsomal monooxygenase system was enzymatically active in the presence of a high concentration of corn oil in the rat serum incubation media. Thus, the failure of the metabolic activation system to potentiate the toxicity of diallate in corn oil is not due to an enzymatic deficiency of the hepatic metabolic activation system. As high concentrations of some organic solvents are known to act directly as embryonic poisons in vitro, rat embryos were cultured in incubation media that contained chloroform (0.5 or 2.5%) dissolved in corn oil. This treatment was found to be severely toxic to embryonic growth and differentiation in vitro. Thus, unlike diallate, the embryonic toxicity of chloroform was fully evident when dissolved in the solvent corn oil. Diallate dissolved in acetone retarded embryo growth and caused dysmorphogenesis. When a metabolic activation system and either corn oil or acetone were used together, the incidence of embryolethality and abnormalities were so high that compound treatment effects were difficult to evaluate.

INTRODUCTION

In vitro whole embryo culture techniques have obvious mechanistic uses in approaching the pharmocokinetic and pharmacodynamic problems of maternal–embryonic interactions. Initially, only direct-acting water-soluble (or gas-phase) compounds could be studied in whole embryo culture.

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This paper has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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(JUNE)

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However, recent methodological improvements have greatly increased the versatility and sensitivity of this technique.

Because embryonic tissues are generally considered to have little or no metabolic activation capacity (e.g., lack of a cytochrome P-450 system), the coupling of adult hepatic metabolic activation systems to whole embryo culture was accomplished (Fantel et al., 1979; Kitchin et al., 1981a, b). This metabolic capacity allows for the direct detection of proteratogens and protoxins like cyclophosphamide (Fantel et al., 1979; Kitchin et al., 1981a, b). An alternative indirect approach uses *in vivo* activation of compounds to produce teratogenic activity in the serum of rats (Klein et al., 1980) or humans (Chatot et al., 1980). A third approach exposes rodent embryos to chemicals *in vivo* followed by an *in vitro* culture period (Beaudoin and Fischer, 1981). Recently, Filler and Lew (1981) have shown that preimplantation mouse embryos have a small but measurable ability to metabolize benzo[a]pyrene.

Subsequently the dose-response relationship for the toxic and dysmorphogenic effects of four water-miscible solvents (dimethyl sulfoxide, ethanol, acetone, and Tween 80) and two water-immiscible solvents (corn oil and acetone/corn oil) on explanted embryos have been described (Kitchin and Ebron, 1982). The ranking of these six solvents in order of their increasing deleterious effect is: corn oil<acetone/corn oil<DMSO< ethanol, acetone<Tween 800. Embryos grew and differentiated well in suspensions of corn oil (2.5–10.0%) in rat serum. All water-miscible solvents were toxic or dysmorphogenic in concentrations between 0.5 and 2.5%. A remaining problem in further developing techniques of *in vitro* embryo culture is to simultaneously expose embryos to water-insoluble compounds in the presence of a functioning metabolic activation system.

In this report, experiments were performed in an attempt to develop an integrated water-insoluble chemical delivery/metabolic activation system for use in whole embryo culture studies. Diallate was selected as a model substance because of its limited solubility in water and its requirement for metabolic activation. Diallate itself is not highly mutagenic, but a diallate metabolite, 2-chloroacrolein, is highly mutagenic (Delorenzo et al., 1978; Schuphan et al., 1979). Corn oil was selected as the first solvent to be tested with metabolic activation. The metabolic activity of hepatic microsomes in rat serum containing large amounts of corn oil was demonstrated. Additionally, corn oil was used as a nontoxic solvent to deliver the water-insoluble compound chloroform to explanted embryos *in vitro*. The results are interpreted with respect to the utility and present limitations of *in vitro* embryo culture techniques for an *in vitro* embryotoxicity/teratogenicity screening procedure and for mechanistic uses in experimental teratology.

METHODS AND MATERIALS

Rat Embryo Culture

On d 10.5 postcoitus (sperm positive is d 0.5), pregnant Sprague-Dawley rats obtained from Charles River, Inc., were sacrificed by guillotine and the abdominal cavity was opened to expose the gravid uterus. Under aseptic conditions, conceptuses were removed from the uterus and collected in petri dishes containing Trowell's T8 medium. The decidual tissue and Reichert's membrane were removed by the techniques described by Sanyal and Wiebke (1979). Whenever possible, embryos harvested from the same dam were put in different treatment groups for culturing. Immediately centrifuged whole rat serum was prepared, stored at −20°C, filtered, and heat inactivated as described previously (Kitchin et al., 1981a, b). Antibiotics (50 IU penicillin/ml and 50 μg streptomycin/ml) were added to the culture serum.

Each explant was individually cultured for 48 h in a 30-ml serum bottle containing 5 ml culture medium and a mixture of gases (5.0% CO_2, 95% air on d 11, followed by 5.0% CO_2, 40.0% O_2, and 55.0% N_2 on d 12). Each culture bottle was rotated on a roller culture assembly at 60 rpm in a 37°C incubator. At the end of the culture period, explants were removed from the culture vessel and observed microscopically. Embryos without a beating heart of functioning yolk-sac circulation were classified as dead and excluded from morphological scoring. The explants were evaluated for growth and differentiation using criteria that included increased yolk-sac diameter, crown–rump length, and number of somites. Some of the embryos and yolk sacs were stored at −20°C for later quantification of DNA (Boer, 1975) and total protein using the Lowry procedure (Lowry et al., 1951). In the assessment of morphology, the embryonic flexion, heart, neural tube, brain development, the otic, optic, and olfactory systems, branchial bars, mandibular and maxillary processes, and fore and hind limb buds were examined. Morphogenesis was scored normal or abnormal as compared to untreated controls.

Liver Microsomes

Washed microsome were isolated from adult (Charles River) male rats of the Sprague-Dawley (CD) strain by standard subcellular fractionation techniques (Kitchin and Woods, 1978). The final microsomal pellet was homogenized in ice-cold rat serum and used fresh on the day of preparation. Some enzyme activities such as hepatic microsomal biphenyl 2-hydroxylation are largely lost when either postmitochondiral supernatant (S-9) or microsomal fracitons are frozen and stored (Dent et al., 1981). The enzymatic ability of the added microsomes (0.4–0.5 mg protein/ml) to

N-demethylate aminopyrine was determined by the ^{14}C method of Poland and Nebert (1973). A NADPH concentration of 1.0 m*M* was used for all embryo culture studies. The indicated concentrations of corn oil and a gas phase of 5% CO_2, 95% air was used in the enzyme assays. Product recoveries were checked by using [^{14}C] formaldehyde as a standard.

Chemicals and Statistics

Diallate (*S*-2,3-dichoroallyl diisopropyl thiocarbamate, 99.7% pure) was obtained from the Pesticide Repository of the U.S. Environmental Protection Agency, Research Triangle Park, N.C. [^{14}C] Aminopyrine and [^{14}C] formaldehyde were obtained from Amersham Searle (Arlington Heights, Ill.). Carrier arninopyrine was purchased from Aldrich Chem. Co. (Milwaukee, Wis.). The solvents corn oil (A&P Tea Co.), acetone (Burdick & Jackson Lab., Muskegon, Mich.), and chloroform (Fischer Scientific Co., Pittsburgh, Pa.) were purchased from the indicated suppliers.

Quantitative data are represented as mean ± SEM for each treatment group. Significance was evaluated using analysis of variance procedures (Snedecor and Cochran, 1967). Embryonic DNA content, somite number, and microsomal aminopyrine *N*-demethylase activity were the quantitative variables analyzed by version 79.5 of the Statistical Analytical System (SAS, 1979). Enumerative data (the number of dead and abnormal embryos) are expressed as the incidence of occurrence; statistical significance was calculated using Fischer's exact test (Goldstein, 1964). The level of statistical significance obtained is indicated in each of the figures and tables.

RESULTS

A dose of 3000 μM diallate in corn oil reduced rat embryo DNA content but not somite number (Fig. 1 and 2). Lower doses of diallate in corn oil did not produce deleterious effects in the final embryonic DNA content or somite number. In general, embryos grew less well in corn oil and the activation system together than in corn oil alone. This pattern is particularly clear in the embryo DNA content (Fig. 1).

Diallate in corn oil did not lead to dose-related embryolethality during the 48-h culture period. More embryolethality occurred with metabolic activation than without it, and the basal incidence of embryolethality is too high to be a satisfactory control for toxicological or teratological studies. In live embryos cultured with diallate, between 20 and 100% of the embryos were classified as abnormal. However, the control incidence of embryonic abnormalities (28 and 60%) is too high to be an acceptable control. Diallate dissolved in corn oil did not show a clear dose-related effect in either embryolethality or abnormality.

In studies with water-miscible solvent, acetone, different results were obtained. With a metabolic activation system, final mean enbryonic values of 64.3, 71.4, 44.1, 53.8, and 8.4 μg DNA/embryo were obtained in em-

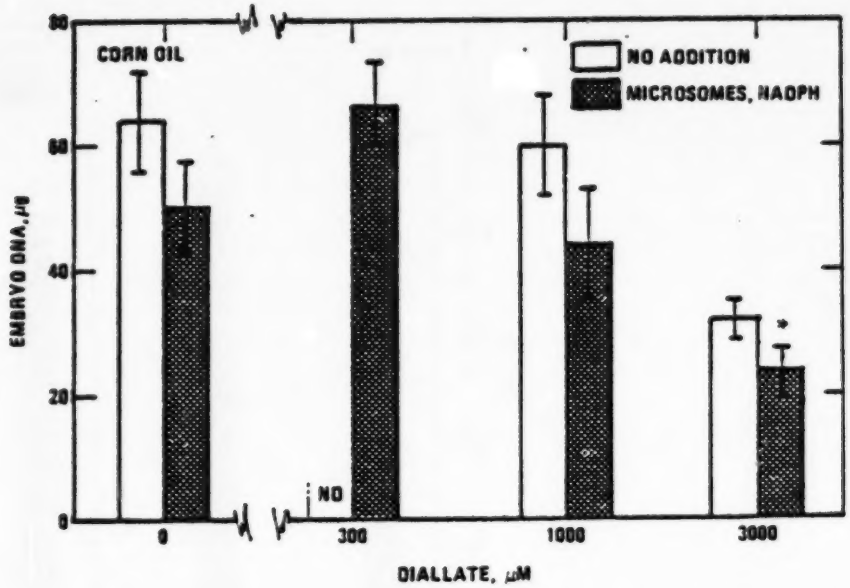

FIGURE 1. Day 10.5 rat embryos were cultured *in vitro* for 48 h in serum containing diallate in corn oil. Either rat hepatic microsomes and NADPH or no addition was added to the cultures. The means ± SEM of total embryo DNA for 5–9 enbryos are displayed.

bryos cultured with 0, 100, 300, 1000, or 3000 μM diallate in acetone, respectively. Because little growth and morphogenesis occurred in this treatment group, the embryonic somite number could not be counted. Increasing concentrations of diallate in acetone did not cause embryolethality but did lead to dose-related dysmorphogenesis. Without the metabolic activation system, 14, 83, 35, and 86% of the cultured rat embryos developed abnormally after exposure to 0, 100, 300, and 1000 μM diallate in acetone. As with the corn-oil data, embryos cultured with a metabolic acitvation system and acetone had a high level of embryonic abnormalities in the absence of any added diallate. With metabolic activation, increasing concentrations of diallate in acetone caused 40, 50, 55, 83, and 100% abnormalities at diallate concentrations of 0, 100, 300, 1000, and 3000 μM, respectively.

Morphologically swollen mandibles and hemorrhage between the fore and hind limb buds were the most common developmental abnormalities due to exposure to diallate in corn oil. In embryos exposed to diallate in acetone, swollen mandibles, hemorrhage in the mandible, and edema in the head region were observed.

Since in the presence of a metabolic activation system 3000 μM diallate was less toxic in corn oil (2.5%) than in acetone (0.1%), we determined

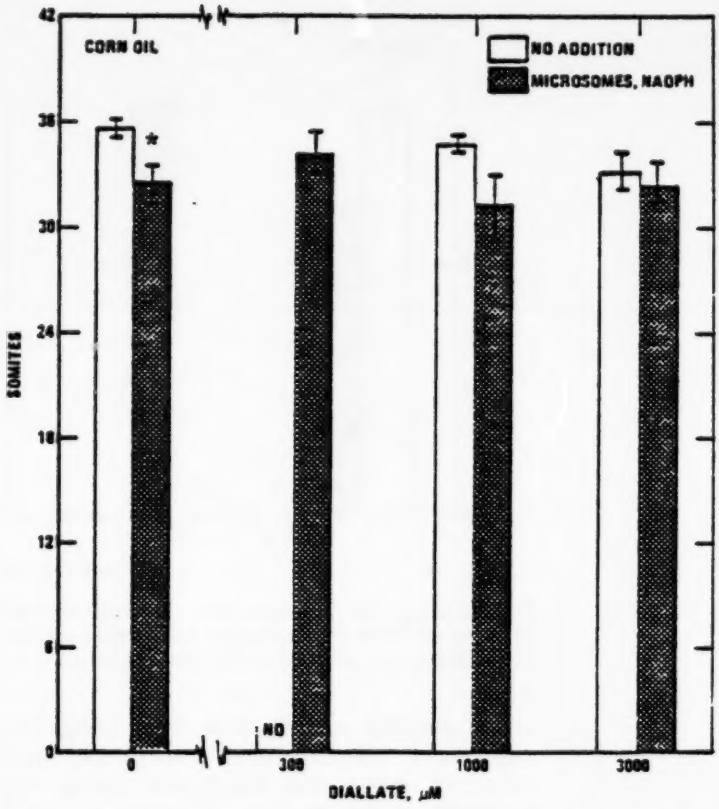

FIGURE 2. Day 10.5 rat embryos were cultured *in vitro* for 48 h in serum containing diallate in corn oil either with or without a metabolic activation system. The means ± SEM of 5–14 embryo somite number determinations are shown.

whether the microsomal mixed function oxidase system was metabolically active in the presence of high concentration of corn oil in serum. Aminopyrine *N*-demethylase activities were 3.92, 3.06, 2.97, and 3.57 nmol formaldehyde/mg protein·min in serum containing 0, 0.1, 0.5, and 2.5% corn oil (Table 1). Thus the activity of hepatic microsomal aminopyrine *N*-demethylase was not inhibited by the presence of corn oil in concentrations up to 2.5%.

We had established that organic solvents are toxic to embryo growth and differentiation *in vitro* (Kitchin and Ebron, 1982). With all solvents except corn oil, the toxic concentrations are roughly equivalent (Kitchin and Ebron, 1982). As diallate's embryotoxicity was not apparent when it was dissolved in corn oil, experiments were performed to assess the embryotoxicity of chloroform (a toxic water-insoluble organic solvent)

TABLE 1. Microsomal Enzyme Activity with High Corn Oil Concentrations

% Corn oil in serum	Aminopyrine *N*-demethylase activity[a] (nmol formaldehyde/mg protein•min)
0	3.92 ± 0.64
0.1	3.06 ± 0.56
0.5	2.97 ± 0.31
2.5	3.57 ± 0.52

[a] [^{14}C] Aminopyrine was added to rat serum media containing male rat hepatic microsomes (0.4–0.5 mg/ml). The formation of [^{14}C] formaldehyde was determined by the method of Poland and Nebert (1973). No comparisons are statistically significant when compared to the controls. The means ± SEM for six determinations are given.

dissolved in corn oil. Chloroform dissolved in corn oil was toxic to developing rat embryos *in vitro* (Table 2). At a concentration of 0.5%, embryo growth and viability were significantly reduced. At 2.5% chloroform, all cultured rat embryos were dead and morphological parameters were unscorable.

DISCUSSION

This report describes the simultaneous use of water-insoluble chemical delivery system and a metabolic activation system. Schematically, this is shown in Fig. 3. Four types of compounds can be distinguished: (a) water-soluble direct acting toxins—e.g., 2,4,6-triethylenimino-1,3,5-triazine (nitrogen mustard) (Sanyal et al., 1978, 1981), $HgCl_2$, CH_3HgCl (Kitchin et al., 1984); (b) water-soluble compounds that require metabolic activation, e.g., cyclophosphamide (Kitchin et al., 1981 a, b); (c) water-insoluble compounds that act without metabolic activation, e.g., chloroform; and (d) water-insoluble compounds that may require metabolic activation, e.g., diallate.

From prior work, it is obvious that cyclophosphamide is more toxic and dysmorphogenic in the presence of a metabolic activation system

TABLE 2. Effect of Chloroform Dissolved in Corn Oil on *in Vitro* Rat Embryo Growth and Differentiation[a]

Treatment	Embryonic DNA (μg)	Somites	Dead embryos	Abnormal embryos
Untreated controls	67.0 ± 3.7	35.0 ± 0.24	0/6	0/6
Corn oil (2.5%)	55.1 ± 6.2	35.2 ± 0.49	0/6	1/5
CCl_3H (0.5%) in corn oil	22.4 ± 5.3**	24.0 ± 3.84*	4/6*	1/2
CCl_3H (2.5%) in corn oil	NM	NM	6/6**	NM

[a] Explanted d 10.5 rat embryos were cultured for 48 h in serum media containing the indicated additions. Six embryos were in each treatment group. NM = not measurable; * = $p < .05$ and ** = $p < .01$, versus corn oil controls. Untreated and corn oil controls were not significantly different.

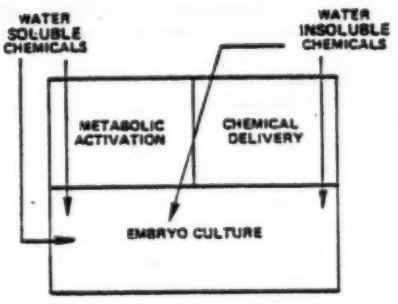

FIGURE 3. *In vitro* toxicodynamic embryo culture methodologies. With the development of chemical delivery systems, either water-soluble or -insoluble compounds may be studied *in vitro*. A metabolic activation system may also be added to the embryo culture system. The arrows illustrate the different ways in which the full experimental capabilities of whole embryo culture can be utilized with four different types of chemicals: water-soluble, direct acting; water-soluble, metabolic activation required; water-insoluble, metabolic activation required; and water-insoluble, direct acting.

than without one (Kitchin et al., 1981 a, b). However, no major effect of the metabolism system is seen in the present data set. A similar lack of a metabolism system effect was obtained with cyclohexylamine and saccharin (Kitchin and Ebron, 1983). The metabolism system by itself sometimes has a small adverse effect on growth and differentiation. Because of this, Schmid et al. (1981) lowered the NADPH concentration in the metabolic activation system to 0.5 m*M*. With corn oil the combined effects of diallate and the microsomal activating system are no more than additive. This lack of potentiation is not due to a failure of the microsomal monooxygenase system to function in the presence of high concentrations of corn oil. Aminopyrine *N*-demethylase activity was changed little by the addition of 0.1, 0.5, or 2.5% corn oil to whole rat serum (Table 1). Previous work has established that microsomal aminopyrine *N*-demethylase activities are similar in either whole rat serum or 0.05 *M* Tris-HCl buffer (Kitchin et al., 1981b).

Diallate was more toxic in acetone than corn oil. This difference was clearly seen at the maximum concentration of diallate tested, 3000 μM. The reason for this difference is unknown. Further studies with acetone or other water-miscible solvents are indicated by this finding. It may be corn oil/serum suspensions partly partition the diallate out of the acqueous phase, but no experimental evidence is available in support of this possibility. In fact, chloroform dissolved in corn oil was quite toxic to developing rat embryos. At the two concentrations tested (0.5 and 2.5%), chloroform was more toxic than any of the previously examined four organic solvents (Kitchin and Ebron, 1982). Thus, corn oil did not antagonize or mask the *in vitro* embryotoxicity of chloroform, as it may have done with diallate.

Doses of diallate under 1000 μM were without growth-retardation

effects. At a dose of 3000 μM diallate in corn oil, growth retardation was observed. The high incidences of embryolethality and abnormality in control groups make meaningful interpretation of the diallate/corn oil data difficult.

In acetone, all doses of diallate (100–3000 μM) tested increased the incidence of abnormal embryos. Thus the experimental results with diallate in acetone show embryonic abnormalities to be a more sensitive parameter than growth or viability. Many of the compounds that have been examined in *in vitro* embryo culture have shown dysmorphogenic effects at doses below those that produced either growth retardation or embryolethality—e.g., cyclophosphamide, $HgCl_2$, CH_3HgCl, DMSO, acetone, Tween 80, ethanol, phosphoramide mustard (Kitchin et al., 1981 a, b; Kitchin and Ebron, 1982; Mirkes et al., 1981).

Partial success has been achieved in integrating a water-insoluble compound delivery system with a metabolic activation system for *in vitro* embryo culture techniques. However, serious problems limit the usefulness of described methods. The chief problem is the high background incidence of embryolethality or abnormalities that were noted with the water-insoluble compound delivery system and a metabolism system were combined. A possible solution to the problem may be to lower the NADPH concentration from 1.0 to 0.5 mM. This may reduce the incidence of embryolethality and abnormality. However, in previous studies with cyclophosphamide, lowering the NADPH concentration led to a related reduction in the adverse effects of the test chemical cyclophosphamide. The level of NADPH used may have been a compromise between lower level, which promotes better embryo viability, growth and morphogenesis and a higher level, which allows for more sensitivity via increased rates of cytochrome P-450 catalysis for longer times. Under our conditions of culture, a NADPH-generating system is toxic (Kitchin et al., 1981a).

A second problem is that with corn oil, the least toxic solvent per se, the metabolic activation system did not potentiate the toxic effects of diallate. This might be due to the experimental compound selected, but it was observed that diallate is substantially more toxic when dissolved in acetone than in corn oil. Corn oil did not inhibit the *N*-demethylation of aminopyrine and the embryotoxicity of chloroform. Thus metabolic enzyme inhibition and test chemical partioning have not yet been observed with corn oil.

More work needs to be done on several questions to clarify some interactions between substrate (test compound), metabolism system, solvent, serum, and the embryo. In particular, additional work with corn oil and acetone is desirable to determine whether other substrates in water immiscible solvents will be less toxic than the same substrates dissolved in water-miscible solvents. Second, more experimental work is needed with other possible water-insoluble chemical delivery/metabolic activation/embryo culture systems to determine if there will be metabolic activation

system-dependent differences in embryotoxicity or dysmorphogenicity with other substances.

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